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<p>(21) International Application Number: PCT/SE94/00421 (22) International Filing Date: 6 May 1994 (06.05.94) (30) Priority Data: 9301583-2 7 May 1993 (07.05.93) SE (71) Applicant (for all designated States except US): KABI PHARMACIA AB [SE/SE]; S-751 82 Uppsala (SE). (72) Inventors; and (75) Inventors/Applicants (for US only): ERNST, Joachim [DE/DE]; Genesheimer Strasse 57a, D-4010 Hilden (DE). JANSSEN, Birger [SE/SE]; Roslagsgatan 47, S-113 54 Stockholm (SE). (74) Agents: TANNERFELDT, Agneta et al.; Kabi Pharmacia AB, S-112 87 Stockholm (SE).</p>		<p>(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.</p>
<p>(54) Title: YEAST STRAIN AND METHODS FOR EXPRESSING HETEROLOGOUS PROTEINS IN YEAST (57) Abstract Saccharomyces cerevisiae strain, characterized in that it is defective in O-mannosylation of a hydroxyl group of at least one serine or at least one threonine residue in a protein expressed by the strain. In the preferred strains the protein is heterologous and encoded in a vector. Process for the production of a protein that normally is O-glycosylated by S. cerevisiae using the strain to express the protein.</p>		

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**YEAST STRAIN AND METHODS FOR EXPRESSING
HETEROLOGOUS PROTEINS IN YEAST.**

5 Technical field:

The invention concerns a novel yeast (*Saccharomyces cerevisiae*) strain that performs O-glycosylation of at least one serine and/or at least one threonine residue at a reduced level. The invention also provides novel methods for the production of
10 heterologous proteins, primarily eukaryotic such as mammalian proteins, in yeast cells.

Technical background:

In fungi O-glycosylation is initiated in the endoplasmatic
15 reticulum and consists of the transfer of a single mannose residue from dolichol-phosphate-mannose (Dol-P-Man) to the nascently secreted protein (Haselbeck and Tanner, FEBS lett. 158 (1983) 335-338). In the yeast *S. cerevisiae* further additions of 3-4 mannose residues using GDP-Man as donor can occur in the Golgi system
20 leading to maximal O-glycosyl chain lengths of 5 mannose residues (Sharma et al., Eur. J. Biochem. 46 (1974) 35-41). Linkages between mannose residues at positions 1, 2 and 3 are α 1-2, while α 1-3 linkages exist between mannose residues at positions 3, 4 and 5.

While the *in vivo* attachment sites for O-glycosyl chains on
25 yeast proteins have not been defined, sites on some heterologous proteins secreted by *S. cerevisiae* are known. Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) is partially O-glycosylated in yeast (Ernst et al., Eur. J. Biochem. 203 (1992) 663-667). O-glycosylated hGM-CSF species appear to carry either
30 an extended chain of up to 5 mannoses on serine-9, or single mannose residues simultaneously on serine-9 and threonine-10. Thus, serine-9 is the principal O-glycosyl attachment site in hGM-CSF. In yeast-secreted human insulin-like growth factor (hIGF-I) about 50% of the secreted protein carries a dimannosyl chain on
35 threonine-29 (Gellerfors et al., J. Biol. Chem. 19 (1989) 11444-49). Serine and threonine attachment sites have also been identified in experiments *in vitro* using synthetic peptides as substrates for

yeast mannosyltransferases (Bause and Lehle, Eur. J. Biochem. 101 (1979) 531-40; Strahl-Bolsinger and Tanner, Eur. J. Biochem. 196 (1991) 185-90; Lorenz et al., Eur. J. Biochem. 205 (1992) 1163-67).

Like in hGM-CSF and hIGF-I the O-glycosylated serine or threonine

5 residues in synthetic peptides may be directly flanked at their N-terminal by a proline residue. The glycosylation patterns in mammalian systems and in *S. cerevisiae* differ significantly, particularly with regard to O-glycosylation attachment sites (serine and threonine) and types of carbohydrates involved.

10 Recently, Strahl-Bohlsinger et al (Yeast 8 (1992) S489, the 16th International Conference on Yeast Genetics and Molecular Biology, Vienna (Austria), August 15-21, 1992) described a gene in *S. cerevisiae*, whose mutation reduces the *in vivo* O-glycosylation by about 50%, while the *in vitro* O-glycosylation in the mutant

15 extract is absent. The *mnt1* mutation leads to shortened O-glycosyl chains consisting of only 2 mannose residues; in this mutant a specific α 1-2 mannosyltransferase is defective (Häusler et al., Proc. Natl. Acad. Sci. USA 89 (1992) 6846-50). The *mnt1* mutation is also known as *kre2* and renders *S. cerevisiae* resistant to the

20 action of K1 killer toxin (Häusler et al., Proc. Natl. Acad. Sci. USA 89 (1992) 6846-50). In *mnn1* mutants, a defective α 1-3 mannosyltransferase does not add the terminal 1-3 linked mannose residues in O-, and N-glycosyl chains (Ballou et al., Proc. Natl.

25 Acad. Sci. USA 88 (1991) 3209-12). Other mutations defective in the synthesis of glycosylation precursors, mannose, GDP-Man and Dol-P-Man, also affect both O-or N-glycosylation. Mutations of this type include defects in SEC59, DPM1, SEC53 and PMI genes encoding dolichol kinase, dolichol phosphate mannose synthase, phosphomannomutase and phosphomannose isomerase, respectively.

30 At present it is not clear, if O-glycosylation in yeast is essential for viability. Only mutations abolishing O- and N-glycosylation completely (SEC59, DPM1, SEC53 and PMI) lead to lethality (Heller et al., Proc. Natl. Acad. Sci. USA 89 (1992) 7013-16; Orlean et al., J. Biol. Chem. 163 (1988) 17499-507; Kepes and Schenkman, J. Biol.

35 Chem. 163 (1988) 9155-61; Smith et al., Mol. Cell. Biol. 12 (1992) 2924-30).

An Article in J. Protein Chem. 9:95-104 (1990) by Elliot et al. describes the purification and properties of yeast-produced hIGF-1. The hIGF-1 protein has been mutated to reduce O-glycosylation. Site-directed mutagenesis was used to convert Thr 29 to Asn29 which reduced but not eliminated IGF-I glycosylation. It thus describes alterations in the IGF-I protein itself, and not the host cell.

EP 314096 concerns yeast mutants that are defective in the addition of outer N-glycosyl chains. EP 276846 discloses the biological activities of various forms of GM-CSF lacking sites for N- and/or O-glycosylation. This patent describes mutants of the expressed protein GM-CSF, and not the host mutants.

Problems solved by the invention:

The unique glycosylation patterns of yeast strains have in many cases a negative impact on the yield obtained of the desired form of heterologously produced proteins. Moreover extra precautions during purification must be applied in order to remove undesired forms of the produced protein.

Figure 1 A and B shows reversed phase HPLC chromatograms.

Objectives of the invention:

One objective of the invention is to provide *S. cerevisiae* expressed proteins, in particular heterologous proteins, that are minimally O-glycosylated at one or more particular serine and/or threonine attachment site(s). An additional objective is to provide a production method for these proteins and a means (a mutated *S. cerevisiae* strain) for the method. In addition the invention will provide *S. cerevisiae* homologous proteins that are minimally O-glycosylated at a serine or threonine site.

The invention:

The *S. cerevisiae* strain of the invention is characterized in that it is defective in initiating O-mannosylation of the hydroxyl group of at least one residue selected from serine and threonine in a protein expressed by the strain. This is probably due to a defect

in the recognition of serine, or threonine acceptor sites, or in the mannosyltransferase activity required to glycosylate these sites. A serine and/or threonine specific O-mannosyltransferase may be missing or altered in activity. Other aspects of glycosylation are similar to wild-type glycosylation, e.g glycosylation performed by strain YE-465. Thus, further attachment of saccharide units to a monomannosylated serine or threonine residue and/or N-glycosylation and/or *in vivo* synthesis of necessary carbohydrate intermediates are in most cases normal as determined by the procedures given in the experimental section.

Particularly important modes of the invention are transformed *S. cerevisiae* strains comprising an expression vector containing a gene for a heterologous, in particular mammalian, protein, such as hGM-CSF or hIGF-I. It follows that in these modes of the invention, the mutation causing decreased O-glycosylation is in a yeast chromosomal gene.

A second main aspect of the invention is a method for producing a protein, often a heterologous, in particular mammalian, protein comprising culturing the inventive *S. cerevisiae* strain and recovering the protein from the fermentation medium or from the cells. It is conceivable that conventional *S. cerevisiae* culturing methods and conventional recovering methods may be used.

Best Mode at the priority date:

The greatest advantage based on today's knowledge for the inventive *S. cerevisiae* strains and production method is obtained for proteins containing one or more serine or threonine residue(s) that are recognized as O-glycosylation sites by wild-type strains of *S. cerevisiae* (containing a vector expressing such a protein) but not by mammalian systems such as in humans. In other words the most preferred *S. cerevisiae* strains of the invention contain an expression vector (e.g. plasmid) for a mammalian, in particular human, protein that is not O-glycosylated by its original species but O-glycosylated by for instance YE-449 containing the appropriate expression plasmid for the protein. The most preferred strain has the same mutation as the M195 strain (see below). The most preferred heterologous protein is hIGF-I.

EXPERIMENTAL PROCEDURES

5 Strains and growth conditions: The starting strain for mutagenesis was *S. cerevisiae* YE-465 which consists of host strain YE-449 (Biogen) (*MAT α leu2 ura3-52 prb1-1122 pep4-3 cir⁰*) carrying plasmid p539/12 (Gellerfors et al., J. Biol. Chem. 264 (1989) 11444-49). p539/12 is an expression plasmid for hIGF-I
10 that leads to the secretion of biologically active hIGF-I into the growth medium of yeast transformants.

Mutagenesis: Ethyl methane sulfonate (EMS) was used to mutagenize strain YE-465. Treatment of a log-phase culture for 120 min and 180 min with 2.5% EMS yielded killing rates of 81%
15 and 91%, respectively. The 180 min culture was plated for single colonies on minimal medium. Single colonies were inoculated with a needle into 2 ml production medium containing 4% casamino acids in reagent tubes and grown for 3 days at 30°C. One ml of the culture supernatant was analysed by concanavalin A blotting, or
20 immunoblotting using an anti-hIGF-I antibody.

 To lose the expression plasmid in putative mutants they were grown non-selectively in YPD medium (Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Lab.) Cold Harbor, N.Y. (1986) 163), followed by the analysis of single isolates for the Ura⁻
25 phenotype. While mutants are named "M", cured mutants were given the designation "CM". Cured mutants (and YE-449 as control) were retransformed with p539/12, or with a secretion vector for hGM-CSF, pER545/4. pER545/4 is a derivative of pER562 (Ernst et al., Bio/Technology 5 (1987) 831-34). Transformants were first grown
30 in selective minimal medium (Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Lab.) Cold Spring Harbor N.Y. (1986) 164); this culture was used to inoculate production medium containing 4% casamino acids (Ernst et al., Bio/Technology 5 (1987) 831-34) and the culture was grown for 2-3 days at 30°C to
35 an OD_{600nm}=10. Cells were removed by centrifugation; for hIGF-I expressing transformants the culture supernatant was concentrated 25-fold by Trichloro acetic acid (TCA) precipitation

prior to electrophoresis followed by concanavalin A and immunoblotting procedures.

Blotting procedures: Glycoproteins in the culture medium were identified by concanavalin A blotting (Clegg, Anal. Biochem. 127 (1982) 389-94). For this purpose 20 μ l of the TCA-concentrated medium was separated by SDS-PAGE (17,5% gel), transferred to nitrocellulose and stained as described. For immunoblottings proteins were transferred to membranes (Immobilon-P, Millipore) and reacted with the monoclonal anti-hIGF-I antibody 5B3 diluted 1:1000, followed by treatment with peroxidase-coupled goat anti-mouse IgG antibody diluted 1:2000 (Jackson Immuno Research, USA). For the detection of hGM-CSF a rabbit polyclonal anti-hGM-CSF antibody (kindly supplied by Glaxo Institute for Molecular Biology, Geneva) was used as first antibody (diluted 1:100), followed by alkaline phosphatase-coupled goat anti-rabbit IgG antibody (Dianova, Germany) diluted 1:5000.

Chromatography procedures: Reversed phase high performance liquid chromatography (HPLC) was performed with a diphenyl silica analytical column equipped with a C4-silica precolumn. The monomeric hIGF-I forms were eluted with an acetonitrile-trifluoro acetic acid (TFA) gradient containing 0.1% (v/v) TFA. The hIGF-I forms were eluted at around 25 % (v/v) acetonitrile.

Other procedures: Standard procedures were used for genetic analyses and crosses of mutants to laboratory strains S150-2B (*MAT α leu2-3,112 ura3-52 trp1-289 his3-1*) and BJ1991 (*MAT α ura3-52 leu2 trp1 prb1-1122 pep4-3 gal2*) (Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Lab.) Cold Spring Harbor, N.Y. (1986) 17-27). Hygromycin B-sensitivity was analyzed on antibiotic gradient plates (gradient from 0 to 200 μ g/ml). Resistance/sensitivity to killer toxin K1 was assayed as previously described (Sherman et al., Methods in Yeast Genetics (Cold Spring Harbor Lab.) Cold Spring Harbor N.Y. (1986) 57-60) using strain RC1777 (*MAT α ade his4C (KIL-k1)*).

RESULTS

Screening for O-glycosylation mutants: Out of 600 mutagenized isolates 8 putative mutants were isolated. To exclude the possibility that the mutagenesis had affected the hIGF-I gene or other genetic material present on the expression plasmid we obtained derivatives of the mutants that had lost their plasmid during non-selective growth. When these cured mutants were transformed with the expression plasmid, an identical phenotype as in the original mutant was obtained. Two of the identified mutants, M195 and M38, were characterized in more detail, as described below.

Characteristics of M195: Mutant strain M195 secretes similar amounts of hIGF-I into the growth medium as compared to the parent production strain YE-465 according to SDS-PAGE and immunoblotting. In contrast, we consistently detected lower concanavalin A-reactivity with hIGF-I secreted by M195, than with hIGF-I secreted by YE-465 indicating reduced O-glycosylation at threonine-29. This phenotype was not due to mutations in the expression vector since the cured retransformed mutant (CM195) also showed reduced hIGF-I O-glycosylation. SDS-PAGE also showed several minor bands of somewhat larger size than IGF-I. These bands represent material O-glycosylated on serine-69, adding to the evidence that the mutation in strain CM195 affects O-linked threonine glycosylation but not serine glycosylation.

In order to quantify the mutant phenotype we purified and analyzed hIGF-I secreted by strains M195 and YE-465 by reversed phase HPLC. HPLC-results for culture media from strain YE-465 and M195 are represented in Figure 1 (For legends see after Results). The chromatograms show four main peaks for monomeric hIGF-1 corresponding to O-glycosylated misfolded hIGF-1, non-glycosylated misfolded hIGF-1, O-glycosylated correctly folded hIGF-I, non-glycosylated correctly folded hIGF-I (i.e. the product). Misfolding of hIGF-1 occurs due to improper disulfide formation from cysteine (Axelsson et al., Eur. J. Biochem. 206 (1992) 987-994). The results clearly demonstrate that the amount of O-glycosylated hIGF-I is reduced and the amount of non-glycosylated hIGF-I is increased for M195 compared to YE-465.

In order to test if strain M195 is also defective for protein O-glycosylation at serine residues, we cured strain M195 of its hIGF-I expression plasmid (resulting in strain CM195) and transformed an expression plasmid for hGM-CSF (pER545/4) into the cured strain. No differences in the glycosylation pattern was found between a wild-type transformant (YE-449(pER545/4)) and the mutant transformant (CM195(pER545/4)) as revealed by SDS-PAGE followed by immunoblotting. This result suggests that serine glycosylation is not affected in M195. On the other hand, N-glycosylation of hGM-CSF also does not seem to be affected in M195, a finding that is confirmed for yeast invertase (see below).

Strain M195 does not show any morphological abnormality, nor does it display temperature sensitivity, or sensitivity to high osmolarities in the growth medium. M195 is as sensitive as the parent YE-465 for killer toxin K1 and 5 mM vanadate. The only detectable phenotype other than its O-glycosylation defect that appears associated with strain M195 is an increased sensitivity for the aminoglycoside antibiotic hygromycin B. While strain YE-465 grows well in the presence of 60 μ g/ml hygromycin B, M195 is completely inhibited. The *mn9* mutation that affects N-glycosylation also is hygromycin B sensitive but resistant to 5 mM vanadate (Ballou et al., Proc. Natl. Acad. Sci. USA 88 (1991) 3209-12). To ascertain that the increased hygromycin B sensitivity in M195 was associated with the observed glycosylation defect we crossed M195 to the wild-type strain S150-2B and examined the haploid progeny. Although many of the segregants of this cross failed to grow we were able to examine hIGF-I O-glycosylation in two hygromycin B sensitive haploid segregants by HPLC analysis and found reduced O-glycosylation in both strains. This finding suggests that reduced O-glycosylation and hygromycin B-sensitivity are due to defects in the same gene.

Characteristics of M38: Secretion of hIGF-I by mutant strain M38 neither shows quantitative, nor qualitative differences compared to the parental strain YE-465; in particular hIGF-I is modified by O-glycosylation to approximately the same extent in M38 and YE-465 as revealed by SDS-PAGE followed by immunoblotting. However, differences in the glycoprotein staining

pattern by concanavalin A demonstrate that several secreted glycoproteins are reduced in size in M38. In particular, a prominent protein of 35 kDa is missing; instead, a 27 kDa glycoprotein of equal intensity is detected.

5 In a wild-type strain of *S. cerevisiae* transformed with an hGM-CSF expressing plasmid immunoblotting on SDS-PAGE of secreted forms of hGM-CSF give bands at 14.5 kDa (unglycosylated), 15.5 kDa (O-glycosylated) and 50 kDa (N-glycosylated) (Ernst et al., Eur. J. Biochem 203 (1992) 663-67). To
10 assess if glycosylation of hGM-CSF is affected, we isolated a plasmid-free derivative of M38. This strain, CM38, was transformed with the hGM-CSF expression plasmid pER545/4 and the hGM-CSF secreted by this transformant was analyzed by immunoblotting. The unglycosylated 14.5 kDa form of hGM-CSF and
15 the heterogeneous N-glycosylated 50 kDa form of hGM-CSF occur both in CM38(pER545/4) and control strain YE-449(pER545/4). However, the O-glycosylated 15.5 kDa form of hGM-CSF is missing in CM38(pER545/4). This result suggests that the defect in M38 is different from the defect in M195, although both defects affect
20 aspects of O-glycosylation in *S. cerevisiae*.

O-glycosylation of chitinase: The O-glycosylation of a homologous yeast protein, chitinase, which is extensively O-glycosylated, was examined in the yeast strains. In YE-465 chitinase appeared on a SDS-PAGE as a band of an approximate
25 molecular weight of 110 kDa. In the mutant strain M195 the migration of chitinase was unchanged, indicating no difference in the O-glycosylation of this homologous protein.

N-glycosylation of invertase in mutant strains: To examine if any of the putative glycosylation mutants is deficient in N-glycosylation, we analyzed the homologous protein invertase, which is essentially only N-glycosylated. Extracts of the mutants were separated on a non-denaturing acrylamide gel and invertase activity was visualised by an activity stain. For two control
30 strains (*mnn9* and *mnn1*) invertase migrates further than for the strain YE-465 and the mutants. By immunoblotting after SDS-PAGE
35 on the same extracts, the defect in the *mnn9* strains is clearly detected leading to a relatively homogeneous protein due to the

lack of outer glycosyl chains, but the production strain and the mutants all express invertase as a heterogeneous glycoform. This result indicates that in the putative mutants including M195 and M38 N-glycosylation is not affected.

5

DISCUSSION

The mutant strains M195 and M38 differ from other mutants that have been shown to be defective in O-glycosylation in *S. cerevisiae*. Because protein N-glycosylation of hGM-CSF and invertase proceed normally in these mutants their genetic defect is different from "unspecific" mutations, such as mutations in SEC59, SEC53, DPM1 or PMI, which affect O-, as well as N-glycosylation. It has been reported recently that defects in the *mnt1* gene lead to a specific shortening of all O-glycosyl chains to two mannose residues (Häusler et al Proc. Natl. Acad. USA 89 (1992) 6846-50). However, in M195 full-length O-glycosyl chains are observed in hIGF-I (although at a low frequency) and in hGM-CSF; in M38 O-glycosylation of hGM-CSF is defective. Thus defects in the *mnt1* gene appear not to be the reason for the mutant phenotype in M195, or M38, although M38 is resistant to the K1 killer toxin, as has been reported previously for *mnt1* strains. A mannosyltransferase has recently been purified from *S. cerevisiae* (Strahl-Bolsinger et al., Yeast 8 (1992) S489, the 16th International Conference on Yeast Genetics and Molecular Biology, Vienna (Austria), August 15-21, 1992) based on *in vitro* O-glycosylation assays using synthetic peptides (Strahl-Bolsinger and Tanner, Eur. J. Biochem. 196 (1991) 185-90). A gene corresponding to the transferase was isolated, the disruption of which leads to loss of *in vitro* mannosyltransferase activity and to a reduction of *in vivo* O-glycosylation to about 50% (Strahl-Bolsinger et al., Yeast 8 (1992) S489, the 16th International Conference on Yeast Genetics and Molecular Biology, Vienna (Austria), August 15-21, 1992). In addition, the disruptant strain shows normal cytology, but forms multiple adherent clumps of cells. Unlike the phenotype of this disruptant, strains M195 and M38 do not form clumps during growth; M195 has no morphological defects, while M38 tends to form rod-like, elongated cells at high

temperature. Also, cell extracts of strains M195 and M38 contain wild-type levels of *in vitro* O-glycosylation (Strahl-Bolsinger, unpublished results). This evidence indicates that neither gene known to affect O-glycosylation is mutated in mutants M195 and
5 M38.

The genetic evidence obtained here and in previous studies strongly suggests that O-glycosylation in *S. cerevisiae* is a complex process that requires multiple cellular factors. The experiments by Strahl-Bolsinger et al., (Yeast 8 (1992) S849, the
10 16th International Conference on Yeast Genetics and Molecular Biology, Vienna (Austria), August 15-21, 1992) indicate that not all of the mannosyltransferases that are active *in vivo* can be assayed *in vitro*, a result that clearly demonstrates the necessity of a genetic approach. The mutants isolated in the present study
15 may be defective in the recognition of serine, or threonine acceptor sites, or in the mannosyltransferase activity required to glycosylate these sites. Thus, M195 may be defective in mannosylation of threonine residues (as in hIGF-I), but not serine residues (as in hGM-CSF). On the other hand M38 may be defective
20 in mannosylation of serine, but not threonine residues. Further analyses of the isolated mutants including gene cloning and their *in vivo* disruption promise to clarify details of the O-glycosylation process in *S. cerevisiae*.

Legends to Figure 1

25 Reversed phase HPLC chromatograms. (A) culture medium from M195; (B) culture medium from YE-465. Four main peaks representing monomeric hIGF-I are visible in each chromatogram, from left (shorter retention time): O-glycosylated incorrectly folded hIGF-I (mismatched), nonglycosylated incorrectly folded
30 hIGF-I (mismatched), O-glycosylated correctly folded hIGF-I and nonglycosylated correctly folded hIGF-I (desired product). There is a remaining small peak in position (3) for the M195 strain (B). This peak might represent some remaining O-glycosylation on serine-29 of hIGF-I or possibly a completely different form of hIGF-I with
35 similar retention time.

C L A I M S

5

1. *Saccharomyces cerevisiae* strain, characterized in that it is defective in O-mannosylation of one hydroxyl group in at least one residue selected from serine and threonine in a protein expressed by the strain.

10

2. *Saccharomyces cerevisiae* strain according to claim 1, characterized in that the strain is defective in O-mannosylation of a free hydroxyl group of at least one serine residue.

15

3. *Saccharomyces cerevisiae* strain according to claim 1, characterized in that the strain is defective in the O-mannosylation of a free hydroxyl group in at least one threonine residue.

20

4. *Saccharomyces cerevisiae* strain according to any of claims 1-3, characterized in that it is a transformant containing a heterologous gene for the protein to be expressed.

25

5. *Saccharomyces cerevisiae* strain according to claim 4, characterized in that

(a) the expressed protein is derived from a mammalian species, and

30

(b) the O-glycosylation of the protein occurring directly at a hydroxyl group of at least one threonine and serine attachment site differs between the mammalian species and a wild-type strain of *S. cerevisiae*,

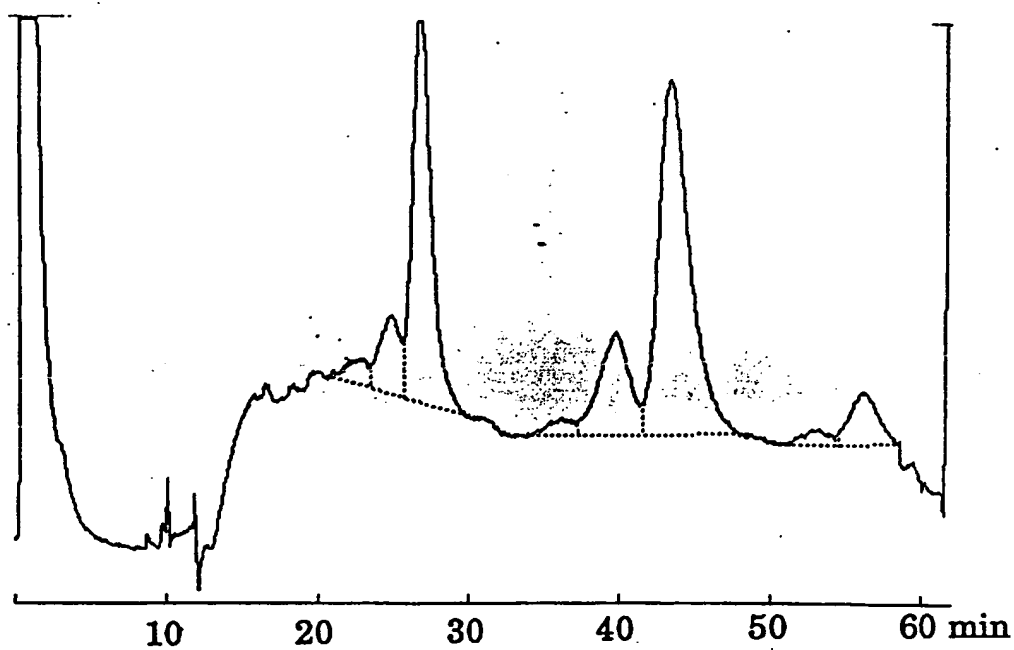
preferably the mammalian species is homo sapiens and the protein is a protein that is not substantially O-glycosylated in homo sapiens.

35

6. *Saccharomyces cerevisiae* strain according to any of claims 4-5, characterized in that the protein to be expressed is human insulin-like growth factor I (hIGF-I).
- 5 7. *Saccharomyces cerevisiae* strain according to any of claims 4-5, characterized in that the protein to be expressed is human granulocyte-macrophage colony-stimulating-factor (hGM-CSF).
- 10 8. *Saccharomyces cerevisiae* strain according to any of claims 1-7, characterized in that the strain performs N-glycosylation that is essentially normal for *Saccharomyces cerevisiae*.
- 15 9. *Saccharomyces cerevisiae* strain according to any of claims 1-8, characterized in that the strain performs essentially normal wild-type attachment of monosaccharide residues to an O-attached monomannosyl serine or O-attached monomannosyl threonine residue.
- 20 10. Process for the production of a protein that normally is O-glycosylated by *S. cerevisiae*, characterized in that a *S. cerevisiae* strain according to any of claims 1-9 is used to express the protein.
- 25 11. Process according to claim 10, characterized in that the protein is human insulin-like growth factor I (hIGF-I) and that the strain carries a gene for said protein.

1/1

A



B

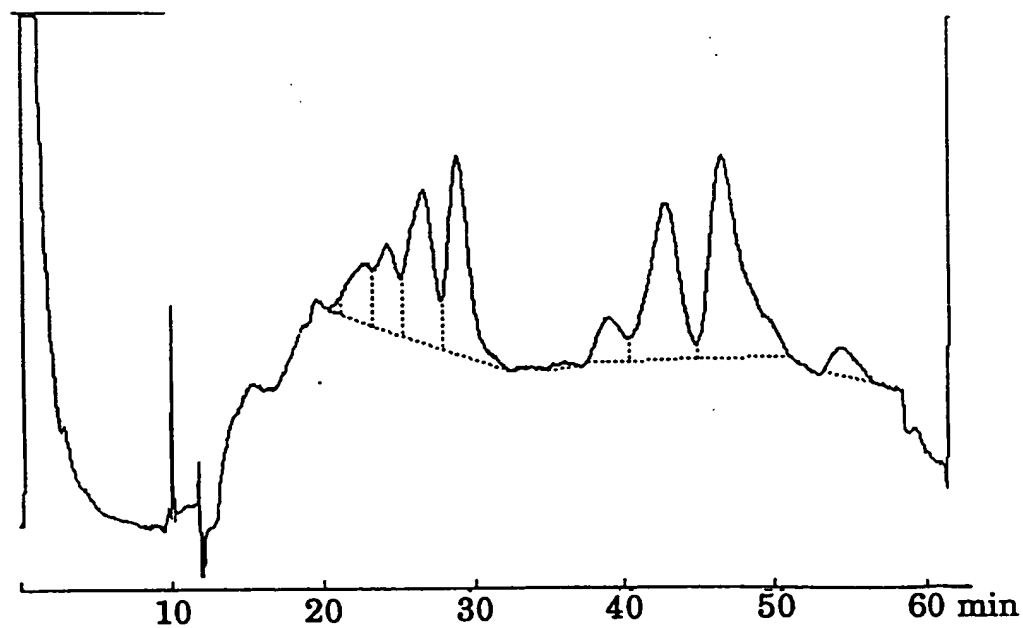


Figure 1

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00421

A. CLASSIFICATION OF SUBJECT MATTER

IPC : C12N 1/18, C12N 15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, EMBASE, BIOSIS, WPIL, US PATENTS FULLTEXT DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X	EP, A2, 0314096 (ZYMOGENETICS INC.), 3 May 1989 (03.05.89), page 2, line 26 - line 28; page 2, line 50 - line 54; page 4, line 52, page 7, line 45 - line 51, claim 1	1-11
A	EP, A2, 0276846 (ZYMOGENETICS, INC.), 3 August 1988 (03.08.88), page 5, line 54 - line 55; page 6, line 37; page 8, line 57 - line 58	1-11

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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Date of the actual completion of the international search

18 August 1994

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 94/00421

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Services, file 155, Medline, Dialog accession no. 07346592, Medline accession no. 90253592, Elliott S et al: "Yeast-derived recombinant human insulin-like growth factor I: production, purification, and structural characterization", J Protein Chem Feb 1990, 9 (1) p 95-104</p> <p>— —————</p>	1-11

INTERNATIONAL SEARCH REPORT
Information on patent family members

02/07/94

International application No.
PCT/SE 94/00421

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0314096	03/05/89	AU-A- 2435688 DE-D, T- 3887082 JP-A- 2000419 US-A- 5135854	11/05/89 28/04/94 05/01/90 04/08/92
EP-A2- 0276846	03/08/88	JP-A- 63301798	08/12/88